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Activity-dependent silencing reveals functionally distinct itch-generating sensory neurons

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Abstract

The peripheral terminals of primary sensory neurons detect histamine and non-histamine itch-provoking ligands through molecularly distinct transduction mechanisms. It remains unclear, however, whether these distinct pruritogens activate the same or different afferent fibers. We utilized a strategy of reversibly silencing specific subsets of murine pruritogen-sensitive sensory axons by targeted delivery of a charged sodium-channel blocker and found that functional blockade of histamine itch did not affect the itch evoked by chloroquine or SLIGRL-NH2, and

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AUTHOR CONTRIBUTIONS

D.P.R. conceived, designed and conducted the behavioral experiments, analyzed behavioral data, and wrote the manuscript. S.G. performed the combined calcium imaging and electrophysiology experiments. J.S. carried out the calcium imaging experiments. H.A.W.P and V.K.R. conducted behavioral experiments and analyzed behavioral data. F.B. contributed to the combined calcium imaging and electrophysiology experiments. B.D. contributed to behavioral experiments and interpretation of behavioral data. S.B.O. gathered behavioral pilot data and gave manuscript advice. B.P.B conceived the silencing strategy, gave technical and conceptual advice, and edited the manuscript. Q.M. provided critical analysis and interpretation of behavioral data, designed behavioral experiments, and contributed to critical revision of the manuscript. A.M.B. conceived the silencing strategy, conceived and designed behavioral experiments and the combined calcium imaging and electrophysiology experiments, gathered behavioral pilot data, supervised the project and wrote the manuscript. C.J.W. conceived the silencing strategy, supervised the project and wrote the manuscript.

Competing Interests Statement

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vice versa. Notably, blocking itch-generating fibers did not reduce pain-associated behavior. However, silencing TRPV1$^+$ or TRPA1$^+$ neurons allowed AITC or capsaicin respectively to evoke itch, implying that certain peripheral afferents may normally indirectly inhibit algogens from eliciting itch. These findings support the presence of functionally distinct sets of itch-generating neurons and suggest that targeted silencing of activated sensory fibers may represent a clinically useful anti-pruritic therapeutic approach for histaminergic and non-histaminergic pruritus.

Itch is a complex unpleasant cutaneous sensation that in some respects resembles pain, yet is different in terms of its intrinsic sensory quality and the urge to scratch. Histamine-mediated itch, as in patients with urticaria, can be effectively treated using histamine receptor antagonists[1]. However, itch accompanying most chronic pruritic diseases, including atopic dermatitis (eczema)[2], allergic itch[3] and dry skin itch[4], is not predominantly mediated by histamine[5]. The G-protein coupled receptors responsive to specific itch-generating ligands are distinct, although at a cellular level, there is overlapping responsiveness of trigeminal and dorsal root ganglia (DRG) neurons to itch-producing pruritogens and pain-producing algogens[6–9]. Histamine-sensitive H1 receptors (H1Rs) generate histamine itch and are expressed by TRPV1$^+$/phospholipase-β-3 (PLCβ3)$^+$ fibers[10,11]. Itch evoked by chloroquine is mediated by Mas-related G-protein-coupled receptor (Mrgpr) A3[6,12], while MrgprC11 is sensitized in dry skin itch[9,13] and activated by pruritogens released from mast cells during allergic itch[3]. Notably, co-activation of TRPV1 and H1R is required to produce histamine itch[14], while MrgprA3- or C11-mediated itch requires co-activation of TRPA1[12] even though each of these TRP channels are canonical nociceptor transducers. *In vitro* calcium imaging experiments find that neurons expressing MrgprA3 also respond to histamine, which is interpreted as indicating a single neuronal path for histaminergic and MrgprA3-dependent itch[6]. Supporting this, ablation of neurons expressing MrgprA3 reduces the scratching evoked by histamine, chloroquine, dry skin, and allergic inflammation[15]. However, others report separate neural pathways mediating histamine and certain types of non-histamine itch[16,17]. Furthermore, while primary sensory neurons of juvenile mice respond to multiple itch mediators, this non-specificity decreases with age[18]. It remains controversial, therefore, if in the adult there are separate afferents that mediate histamine itch and MrgprA3-dependent non-histamine itch. This distinction is clinically important since therapies targeting histaminergic itch fibers might be ineffective for treating non-histaminergic itch if the neurons mediating the two itches are functionally distinct in the adult.

To study if histaminergic and non-histaminergic itch are functionally distinct we adapted a method originally designed for achieving a pain-specific peripheral nerve block[19,20] to selectively silence the peripheral terminals of different subsets of pruritogen- and algogen-responsive primary afferents in an activity-dependent manner. To do this we targeted the charged, membrane-impermeable lidocaine derivative N-ethyl-lidocaine (QX-314) (a sodium channel blocker) through large pore ion channels activated specifically by different algogens and pruritogens.

RESULTS

Targeted Na$^+$ current block of pruritogen-activated neurons

Activation of TRPV1 channels permits entry of QX-314 selectively into dorsal root ganglion (DRG) and trigeminal ganglion nociceptors through the TRPV1 pore to produce a selective block of sodium currents only in TRPV1 expressing nociceptors[19–24]. Here we examined whether histamine-mediated activation of TRPV1 channels[11,14] would allow sufficient QX-314 uptake to suppress sodium channel currents selectively in histamine-responsive trigeminal ganglion neurons. In trigeminal neuron cultures from adult male CD-1 mice we

recorded sodium currents using whole cell voltage clamp from small (<25 μm diameter) neurons that showed an increase in intracellular calcium concentration upon a 60 second bath application of 100 μM histamine (Fig. 1a). In these cells, a subsequent 2.5 minute application of 100 μM histamine together with 5 mM QX-314 significantly and progressively decreased sodium current amplitude with a nearly complete block after 10 minutes (Fig. 1b,c and Supplementary Tables 1 and 2). This decrease was prevented by the TRPV1- channel blocker capsazepine (20 μM) (Fig. 1c). Sodium currents recorded from trigeminal neurons that did not respond to histamine were not affected by co-application of histamine and QX-314 (Fig. 1b,c), indicating that extracellularly applied QX-314 by itself at this dose has no activity. Together with previous studies demonstrating that histamine produces downstream activation of TRPV1 channels[14], our data indicate that QX-314 enters histamine responsive trigeminal neurons when activated by histamine, likely through TRPV1 channels.

We then examined whether we could block sodium currents in chloroquine-responsive trigeminal neurons by co-application of chloroquine and QX-314. TRPA1 channels are activated by chloroquine in these cells[12]. We and others have demonstrated that QX-314 and other cationic organic compounds with a similar molecular weight permeate the TRPA1 pore[23,25]. In trigeminal neurons that showed a robust increase in intracellular calcium after brief chloroquine application (100 μM, 60 seconds) (Fig. 1d and Supplementary Tables 3 and 4), subsequent co-application of 100 μM chloroquine and 5 mM QX-314 significantly decreased sodium currents (Fig. 1e,f). This effect was significantly reduced by pre-incubation with the TRPA1-channel blocker HC-030031 (100 μM) (Fig. 1f). In cells that did not respond to chloroquine, QX-314 and chloroquine had no effect on sodium current (Fig. 1e,f).

Pruritogen-mediated activation of TRP channels (histamine for TRPV1 and chloroquine for TRPA1) permitted, therefore, sufficient entry of QX-314 selectively into particular pruritogen-sensitive trigeminal neurons to block sodium currents only in these neurons (Supplementary Fig. 1).

Selective silencing of pruriceptors *in vivo*

To test if we could selectively silence pruriceptors *in vivo* we used intradermal cheek injections of pruritogens together with QX-314, then characterized pruriceptor function by quantifying ensuing itch behavioral responses (hindlimb scratching of cheek) or pain (forelimb wiping of cheek)[26]. Intradermal injection of histamine (100 μg/20 μl) produced scratching (64.3 ± 7.5 bouts, $n = 6$) that fully resolved within 30 minutes. There was no significant change in scratching (56.0 ± 8.3 bouts, $n = 6$; $P > 0.05$) when histamine was injected together with 1% QX-314. Based on the delayed time course of nociceptor block *in vivo* in response to a combination of capsaicin and QX-314[19], and considering the gradual development of full sodium current block *in vitro* after QX-314 and histamine coadministration (Fig. 1c), we hypothesized that the short duration of histamine-evoked scratching behavior (~25 minutes) was too brief to detect the slow onset blocking effects generated by coadministration of histamine with QX-314. To test this we devised a behavioral model using two sequential intradermal injections, 30 minutes apart, of a pruritogen into the same intradermal cheek-injection site. Identical pruritogen doses were used in each injection. To ensure that both injections were distributed within the same cutaneous area, however, the first dose (conditioning injection) was delivered in 20 μl of vehicle while the second dose (test injection) was administered in 10 μl (Supplementary Fig. 2). Only behavior evoked by the test (second) injection was compared to behavior evoked by other identical test injections, with the experimental variable being the identity of the conditioning (first) injection given 30 minutes earlier.

When histamine alone was given for the conditioning injection (100 μg/20 μl) and again 30 minutes later for the test injection (100 μg/10 μl) at the same site, the amount of scratching evoked by the two injections was not significantly different (Fig. 2a). However, histamine-evoked scratching was effectively abolished 30 minutes after a conditioning injection of histamine and QX-314 together (Fig. 2b), but not when preceded by injection of QX-314 alone (Supplementary Fig. 3a). We conclude that histamine-mediated activation of large pore channels, such as TRPV1[14], permitted uptake of QX-314, to produce a slow (<30 minutes) onset electrical silencing of the histamine-responsive sensory fibers, which then blocked the response to subsequent injection of histamine (Fig. 2b).

Based on our *in vitro* results (Fig. 1d–f) we hypothesized that intradermal administration of chloroquine with QX-314 could block chloroquine itch by permitting selective uptake of QX-314 through chloroquine-mediated activation of TRPA1[12]. Co-injection of QX-314 together with chloroquine (50 μg/20 μl) inhibited scratching produced by subsequent chloroquine (50 μg/10 μl) test injection, while injection of chloroquine alone or QX-314 alone did not (Fig. 2c,d and Supplementary Fig. 3b). We conclude that large-pore channels downstream of chloroquine-evoked MrgprA3 activation, likely TRPA1[12], permitted selective uptake of QX-314 and subsequent electrical silencing of chloroquine-sensitive afferent fibers.

SLIGRL-NH_2 (SLIGRL) acts at MrgprC11 receptors to produce itch[9]. MrgprC11-mediated itch also requires TRPA1 activation to generate scratching behavior[12]. We next explored, therefore, whether SLIGRL-mediated activation of TRPA1 channels is sufficient to introduce QX-314 and inhibit subsequent SLIGRL-evoked itch. SLIGRL (50 μg) evoked scratching bouts were inhibited 30 minutes after injection of QX-314 together with SLIGRL, but not after injections of SLIGRL alone or QX-314 alone (Fig. 2e,f and Supplementary Fig. 3c).

These findings demonstrate that histaminergic and non-histaminergic pruritogen-mediated entry of QX-314 into pruriceptors is sufficient to block activity of itch-generating fibers, as measured by a reduction in scratching behavior on subsequent injection of the same pruritogen.

Pruriceptors are dispensable for non-itch sensations

It is unclear whether fibers that mediate histamine itch contribute to normal chemical, thermal and mechanical pain sensitivity. To explore this, we assessed pain-related behavioral responses to noxious chemical, thermal, and mechanical stimulation after co-injection of QX-314 together with chloroquine (50 μg), histamine (100 μg), capsaicin (0.1%), or AITC (0.15%). Injection of capsaicin in the cheek evoked robust forelimb wiping (indicative of TRPV1-evoked pain) 30 minutes after vehicle (0.9% NaCl) injection (Fig. 3a). Administration of QX-314 together with capsaicin, as expected, abolished subsequent capsaicin-evoked wiping (Fig. 3a). However, capsaicin-evoked wiping was unchanged 30 minutes after injection of QX-314 together with either histamine or chloroquine (Fig. 3a), even though such injections block scratching generated by these pruritogens (Figs. 2 and 4). Injections of histamine, chloroquine, or QX-314 alone did not reduce subsequent capsaicin-evoked wiping (Supplementary Fig. 4a).

Test injection of AITC (0.15%) evoked robust forelimb wiping (indicative of TRPA1-evoked pain) 30 minutes after vehicle (0.9% NaCl) injection (Fig. 3b). AITC-evoked wiping was blocked after a conditioning injection of QX-314 together with AITC (0.15%), but not after conditioning injections of QX-314 together with chloroquine (50 μg), or histamine (100 μg), or by prior administration of QX-314, chloroquine, or histamine alone (Fig. 3b and Supplementary Fig. 4b).

We next asked whether blocking histamine- or chloroquine-responsive fibers affects mechanical or thermal pain sensitivity. Intraplantar co-injection of QX-314 (20 μl) together with histamine (100 μg) or chloroquine (50 μg) had no effect on mechanical sensitivity to von Frey filaments, while co-application of capsaicin (0.1%) and QX-314 abolished mechanical pain responses (Fig. 3c). Likewise, intraplantar injection of QX-314 (20 μl) together with histamine (100 μg) or chloroquine (50 μg) did not alter responses to noxious heat (52° C). Noxious thermal sensitivity was, however, abolished when tested after coadministration of capsaicin (0.1%) and QX-314 (Fig. 3d).

To determine whether histamine and chloroquine itch-generating fibers are dispensable for sensitivity to cold or tactile stimuli, we performed the focal cold plantar assay[27] and the adhesive dot plantar tactile assay[28] 30 minutes after intraplantar injections of pruritogens and algogens together with QX-314 (Fig. 3e,f). Cold sensitivity was unaltered 30 minutes after injections of QX-314 together with histamine (100 μg), chloroquine (50 μg), or capsaicin (0.1%). However, cold sensitivity was significantly reduced after co-injection of AITC (0.15%) and QX-314 (Fig. 3e). We next used latency to attend to an adhesive dot placed on the plantar paw surface as a measure of tactile sensitivity[28]. The latency to attend was unchanged 30 minutes after injections of QX-314 with histamine (100 μg), chloroquine (50 μg), capsaicin (0.1%), or AITC (0.15%), while intraplantar injection of lidocaine (5%) significantly increased the latency (Fig. 3f).

We propose that while histamine and chloroquine activate TRPV1 and TRPA1 expressing pruriceptors, respectively, these fibers are not essential for acute thermal, mechanical and chemical pain sensitivity, which likely involve different or additional subsets of afferents to generate pain-related behavior. Likewise, we conclude that chloroquine- and histamine-sensitive itch fibers are not essential for normal tactile sensitivity.

Distinct fibers mediate histamine and non-histamine itch

We next used the silencing strategy to reveal whether different pruritogens activate the same or unique peripheral afferent pathways by administering test injections (10 μl) of histamine (100 μg), chloroquine (50 μg) or SLIGRL (50 μg), 30 minutes after conditioning injections (20 μl) of histamine (100 μg), chloroquine (50 μg) or SLIGRL (50 μg) with and without QX-314. We hypothesized that if histaminergic and non-histaminergic itch are transmitted by different subsets of afferent fibers, application of histamine together with QX-314 will block histaminergic itch while chloroquine or SLIGRL itch will remain intact, and *vice versa*.

We first examined whether histamine-evoked scratching is affected by the targeted silencing of chloroquine-activated pruriceptors. Histamine-evoked scratching was unchanged 30 minutes after a conditioning injection of chloroquine and QX-314 together (Fig. 4a), even though this treatment blocked subsequent chloroquine itch (Fig. 2b and 4b). Similarly, conditioning injection of SLIGRL with QX-314 (which reverses later SLIGRL-evoked scratching; Fig. 2c and 4c) did not reduce subsequent histamine (100μg/10μl) itch (Fig. 4a).

We then asked if chloroquine itch is affected upon silencing of histamine- or SLIGRL-sensitive itch fibers by injecting chloroquine thirty minutes after administration of QX-314 together with either histamine or SLIGRL. Chloroquine-evoked itch was blocked after coinjection of SLIGRL and QX-314, but not by coadministration of histamine and QX-314 (Fig. 4b). Likewise, SLIGRL-evoked itch was unaffected 30 minutes following injection of histamine with QX-314, but was significantly reduced after injection of chloroquine and QX-314 (Fig. 4c).

It appears that a common afferent population mediates chloroquine and SLIGRL itch, but that this population is functionally distinct from neurons responsible for histamine itch. These findings contrast, however, with prior *in vitro* data showing that all chloroquine-responsive DRG neurons respond to histamine[6]. A possible explanation for this discrepancy is that peripheral terminals of trigeminal neurons differ from DRG neuron cell bodies in terms of their responsiveness to multiple pruritogens. To explore this, we repeated the same silencing strategy on the back below the neck. Treatment with histamine and QX-314 together did not reduce subsequent chloroquine-evoked scratching when compared to treatment with vehicle, while intradermal injection of chloroquine and QX-314 together inhibited subsequent chloroquine-evoked scratching (Supplementary Fig. 5) indicating that facial and somatic itch appear to be similar in terms of the functional independence of histamine and non-histamine itch.

Another possibility is that different pruriceptor subsets exist with high or low sensitivity to either histamine or non-histamine pruritogens. To investigate this we varied the dose of pruritogen given with 1% QX-314 (20 μl) for conditioning injections. Application of a lower dose of histamine (10 μg) together with QX-314 inhibited subsequent histamine-evoked scratching without reversing chloroquine (50 μg) scratching (Fig. 4a,b), essentially identical to the effects following a higher dose of histamine (100 μg) with QX-314. However, increasing the concentration of histamine to 108.7 mM (400 μg/20 μl) with QX-314 for conditioning injections blocked scratching evoked by both histamine and chloroquine (Fig. 4a,b) indicating that an overlap manifests only at very high doses of histamine.

Higher doses of chloroquine (200 μg or 400 μg) together with QX-314 blocked later chloroquine itch behavior, but not scratching evoked by histamine. However, using 96.9 mM chloroquine (1 mg/20 μl) together with QX-314 for conditioning injections blocked subsequent histamine- and chloroquine-evoked scratching (Fig. 4a,b).

These data suggest that histamine and chloroquine generally activate functionally distinct pruriceptor populations, but that these fibers can respond to multiple pruritogens, either directly or indirectly, when they are presented at very high, presumably non-physiological[29,30] concentrations (histamine, 107.8 mM; chloroquine, 96.9 mM).

Histamine and chloroquine act on different sets of neurons

The responsiveness of DRG neurons to multiple pruritogens decreases with age[18]. In juvenile mice, 60–100% of all chloroquine responsive DRG cells also respond to histamine[6,18], while only half of chloroquine-responsive DRG neurons respond to histamine in adolescent mice[18]. We therefore asked whether distinct chloroquine and histamine-sensitive neuronal populations could be revealed in adult (2 to 4-month-old) mice by their sensitivity to pruritogens *in vitro*. MrgprA3 receptors are activated by 10 μM chloroquine and maximally activated by 1 mM chloroquine in HEK293 cells[6]. Murine DRG neurons respond to histamine at doses as low as 10 μM[14]. Here, we examined coincident calcium responses of 564 cultured trigeminal neurons to 10 μM histamine and 10 μM chloroquine. Histamine activated 4.1% (23 of 564) of trigeminal neurons, while 3.0% (17 of 564) responded to chloroquine (Fig. 5a). A majority of chloroquine-and histamine-responding trigeminal neurons responded to only one pruritogen. Among chloroquine-activated cells, 76.5% (13 of 17) responded to chloroquine but not histamine, while the remaining 23.5% (4 of 17) responded to both 10 μM histamine and 10 μM chloroquine. Likewise, trigeminal neurons responding to histamine were largely unresponsive to chloroquine: 82.6% (19 of 23) responded to histamine but not chloroquine, and 17.4% (4 of 23) responded to both histamine and chloroquine.

To investigate whether responsiveness of adult trigeminal neurons to chloroquine or histamine is dependent upon pruritogen dose, we further explored coincident calcium responses to 100 μM histamine and 100 μM chloroquine. Most trigeminal neurons that responded either to 100 μM histamine (4.8% of all cells) or 100 μM chloroquine (4.3% of all cells) did not respond to both (0.9% of all cells) (Supplementary Fig. 6a). Moreover, we also found in DRG neurons from adult mice that the populations of fibers responding to chloroquine and histamine are, as in the trigeminal ganglia, largely distinct (Supplementary Fig. 6b). These data demonstrate that primary afferent populations responding to histamine and chloroquine are largely distinct in adult mice.

The requisite expression of TRPV1 for histaminergic itch[14], and of TRPA1 for chloroquine-evoked itch[12] raises the question: do relative expression patterns of TRPA1 and TRPV1 differ among chloroquine- and histamine-sensitive neuronal populations? To answer this, we exposed cultured trigeminal neurons to 100 μM AITC and 1 μM capsaicin and measured coincident responses of neurons to both agents. Consistent with the described receptor expression patterns for neurons that mediate histamine itch (e.g., H1R and TRPV1)[10,14] and chloroquine itch (e.g., MrgprA3 and TRPA1)[6,12], we found that a majority (16 of 23, 69.6%) of histamine-responsive cells responded to capsaicin (Fig. 5b), and most (9 of 17, 52.9%) chloroquine-activated neurons were sensitive to AITC (Fig. 5c). The inverse relationships were also true: most (14 of 23, 60.8%) histamine-responsive trigeminal cells did not respond to AITC (Fig. 5d), and capsaicin failed to activate a majority (12 of 17, 70.6%) of chloroquine-activated neurons (Fig. 5e).

Roles of TRP channels in histamine and non-histamine itch

The different expression patterns of TRPV1 and TRPA1 among histamine and chloroquine-sensitive neurons *in vitro* (Fig. 5) suggest that targeted silencing of TRPV1 or TRPA1 fibers may differently affect histamine itch versus chloroquine itch. To explore this we administered 20 μl conditioning injections of capsaicin (0.1%) or AITC (0.15%) with or without QX-314 and then administered 10 μl test injections of the pruritogens histamine (100 μg), chloroquine (50 μg) or SLIGRL (50 μg) 30 minutes later at the same site. Injection of capsaicin alone did not significantly reduce subsequent histamine-, chloroquine-, or SLIGRL-evoked scratching (Supplementary Fig. 3). However, when the conditioning injection of capsaicin was administered together with QX-314, the scratching evoked by subsequent histamine injection was abolished (Fig. 6a). Administration of capsaicin with QX-314 also significantly reduced but did not eliminate scratching produced by subsequent injections of chloroquine or SLIGRL (Fig. 6b,c) even though only a third of chloroquine neurons are capsaicin sensitive (Fig. 5e), implying that the $TRPV1^+$ subset of chloroquine-sensitive neurons may have a particularly prominent role in eliciting behavioral itch responses.

When QX-314 was administered together with AITC for the conditioning injection, subsequent SLIGRL-evoked scratching was significantly reduced (Fig. 6c). Likewise, chloroquine-evoked scratching was virtually abolished 30 minutes after injection of QX-314 together with AITC (Fig. 6b). In contrast, injection of QX-314 with AITC did not reduce subsequent histamine-evoked scratching (Fig. 6a). In summary, chloroquine-and SLIGRL-evoked scratching is effectively blocked when $TRPA1^+$ (AITC-responsive) fibers are electrically silenced. Histamine-evoked scratching, on the contrary, is largely abolished when $TRPV1^+$ fibers are electrically silenced but is unaffected when $TRPA1^+$ (AITC-responsive) fibers are blocked. Histamine itch is predominantly associated with $TRPV1^+$ pruriceptor fibers, and non-histamine itch with $TRPA1^+$ fibers.

TRPV1$^+$/TRPA1$^+$ afferents are involved in itch inhibition

Cheek injection of capsaicin normally evokes only a wiping (pain) response without any significant scratching (itch)[26,31]. We found, however, that cheek injection of capsaicin (0.1%, 10 μl) produced significant scratching (itch) together with wiping (pain) when TRPA1-responsive nociceptors were first silenced by conditioning coinjection of AITC (0.15%, 20 μl) with 1% QX-314 (Fig. 7a). Injection of capsaicin (0.1%, 10 μl) 30 minutes after 20 μl injection of AITC (0.15%), capsaicin (0.1%), QX-314, or vehicle (0.9% NaCl) alone produced only wiping (Supplementary Fig. 4a). Capsaicin-evoked scratching following coinjection of AITC (0.15%) with QX-314 was prevented when the conditioning combination included histamine (100 μg) (Supplementary Fig. 3d). These data suggest that a subset of TRPA1$^+$ sensory neurons normally mask capsaicin-evoked itch, and that silencing these neurons allows capsaicin to generate itch-related behavior through uninhibited activation of TRPV1$^+$ histaminergic itch fibers.

When we repeated the experiments using AITC (0.15%, 10 μl) as the test algogen (Fig. 7b) we again observed that algogen-mediated electrical silencing, this time by coinjection of capsaicin (0.1%) and QX-314, resulted in both wiping and scratching upon test injection of AITC (Fig. 7b). The AITC-evoked scratching was largely reversed when chloroquine- and capsaicin-responsive fibers were both silenced by a conditioning injection of chloroquine (50 μg), capsaicin (0.1%) and QX-314 together (Supplementary Fig. 3e). These findings suggest that a subset of TRPV1-expressing sensory neurons normally mask itch following AITC activation of chloroquine-sensitive itch fibers.

Thus, silencing TRPA1$^+$ fibers and silencing TRPV1$^+$ fibers allow capsaicin and AITC, respectively, to evoke scratching (itch) responses. Our interpretation is that there is a subset of peripheral TRPV1$^+$/TRPA1$^+$ neurons that when activated normally inhibit or mask itch via central inhibitory interneurons[32], and whose silencing then allows either capsaicin or AITC to abnormally evoke itch.

DISCUSSION

We selectively inhibited pain- and itch-related behaviors by targeting the membrane-impermeant sodium channel blocker QX-314 into peripheral axon terminals of distinct populations of trigeminal pruriceptors or nociceptors. The specific population silenced was determined by the pattern of activation of particular large pore channels by different pruritogens or algogens; TRPV1 for capsaicin and histamine, TRPA1 for AITC, chloroquine and SLIGRL. We show by electrophysiological recording that activation of these large pore channels by histamine and chloroquine enables sufficient permeation of QX-314 into trigeminal neurons to block sodium currents, as previously shown for capsaicin[19], and that this effect is specific; only pruritogen-activated neurons are blocked. The fact that specific TRP-channel blockers (TRPV1 for histamine and TRPA1 for chloroquine) can prevent permeation of QX-314 indicates that it is TRP channel mediated. This has enabled us to exploit the silencing of different afferents to tease out their functional sensitivity to defined stimuli. This approach differs from interventions that only block a particular receptor (e.g., H1R, H4R)[33] or channel (e.g. TRPA1)[34] in that it targets action potential generation and conduction of the activated axon, and differs from genetic targeted ablation of different sensory neuronal subtypes[35] in that it is temporary with no compensatory changes.

Utilizing this selective silencing strategy we reveal that fibers that mediate histamine and non-histamine itch are functionally separable. Moreover we demonstrate that activation of these itch-generating fibers is not required for eliciting normal responses to acute mechanical and thermal stimuli. Our *in vitro* data confirm the presence of adult sensory neurons that respond only to histamine or only to chloroquine.

Separate afferent lines are described for histamine and cowhage itch[16,36] and a distinct non-histamine itch pathway activated by β-alanine[17]. Our data support, in addition, separate functional pathways for histamine itch and itch mediated by the MrgprA3 and C11 ligands chloroquine and SLIGRL, respectively. The separation of these afferents based on the silencing approach was defined for a broad range of pruritogen concentrations for targeting QX-314 into afferent terminals through activated TRP channels. At extremely high concentrations (>90 mM) of pruritogen *in vivo*, however, an overlap did occur between histamine and chloroquine populations. This cross-activation between the two populations could be secondary to release of endogenous mediators from keratinocytes, mast cells or other non-neuronal cells activated secondary to high-dose pruritogen administration, or it could reflect a very limited sensitivity of the peripheral terminals of histaminergic pruriceptors to chloroquine and SLIGRL, and of the non-histaminergic terminals to histamine. Given that the high concentrations of the opposing pruritogen required to co-activate the separate histamine or non-histamine responsive set of afferents are unlikely to be found in most natural conditions[29,30], we consider it probable that the two sets are functionally distinct and normally act independently.

Our calcium imaging data also confirm the existence of distinct trigeminal and DRG neuron populations that respond to either chloroquine or histamine at a range of doses in adult mice. Prior *in vitro* experiments have shown that MrgprA3 lineage DRG neurons in juvenile (4-week-old) mice respond both to chloroquine (1 mM) and histamine (50 μM)[6]. Similarly, ablation of MrgprA3$^+$ neurons in 5-week-old mice significantly attenuates both chloroquine- and histamine-evoked scratching behavior[15]. However, a recent study that showed that the proportion of DRG neurons responding to both histamine and chloroquine is markedly less in 7 to 9-week-old mice compared to 3 to 4-week-old mice[18]. We interpret these collective data as suggesting that in adult (2 to 4-month-old) mice, Mrgpra3$^+$ neurons are likely composed of pruriceptors with a differential sensitivity to, but not absolute selectivity for chloroquine, and that these afferents normally contribute primarily to non-histaminergic itch. Microneurographic studies in humans have identified a distinct set of histamine-insensitive fibers activated during cowhage-evoked itch[16]. The finding that cowhage spicules activate MrgprA3$^+$ neurons suggests that a common pathway may mediate chloroquine and cowhage itch[15]. However, cowhage spicules act promiscuously on many nociceptor subtypes and may instead trigger itch nonspecifically through focal activation of superficial nociceptor terminals[37].

H1R and MrgprA3 receptors rely on downstream activation of TRPV1 or TRPA1 channels, respectively, to generate itch behavior[11,12,14], which is somewhat counterintuitive since these TRP channels are also activated by algogens (capsaicin and AITC) that normally produce pain. This raises questions both whether there are different subsets of TRPV1$^+$ or TRPA1$^+$ neurons involved in processing pain or itch, and why pain normally predominates. Our data show that histamine itch is mediated by TRPV1$^+$ fibers that do not express appreciable levels of TRPA1, since histamine itch is inhibited by silencing capsaicin-activated fibers, but not by silencing AITC-activated fibers. This correlates with the coincidence of capsaicin and histamine responsiveness in most histamine responsive trigeminal neurons. Chloroquine or SLIGRL itch, in contrast, appears to be mediated mainly by TRPA1$^+$ fibers. But, using capsaicin-induced silencing and trigeminal neuron calcium imaging we show that a subset of these fibers also coexpress TRPV1. TRPV1 is therefore promiscuously expressed in nociceptors and both histaminergic and non-histaminergic pruriceptors.

Electrical silencing of either histamine-sensitive or chloroquine-sensitive primary afferents blocks itch but does not alter a wide range of pain-associated behaviors, suggesting that while these primary afferent fibers are required for itch, as are GRPR$^+$ neurons in the dorsal

spinal cord[35,38], they are not necessary for eliciting acute thermal or mechanical pain, as also demonstrated for the MrgprA3$^+$ population[15]. Our data do not rule out the possibility, though, that activation of some histamine-sensitive or chloroquine-sensitive fibers might be sufficient to produce pain. For example, histamine can evoke pain in rodents and humans[39,40], particularly in bradykinin-sensitized nociceptors[41]. We also find a group of peripheral neurons expressing both TRPA1 and TRPV1 that appear to be involved in a functional inhibition or masking of itch, since silencing either TRPA1$^+$ fibers or TRPV1$^+$ fibers now allows capsaicin or AITC to evoke itch, rather than pain. Consistent with this, when nociceptive sensitivity is reduced by abolishing vesicular glutamate transporter type 2 (VGLUT2)-dependent synaptic glutamate release in nociceptors, intradermal capsaicin injection is also now able to generate scratching[42]. We propose, therefore, that the algogen-evoked itch that follows electrical silencing of contra-algogen-responsive fibers may represent a pain-to-itch synesthesia produced only when TRPV1$^+$ histaminergic pruriceptors or TRPA1$^+$ non-histaminergic pruriceptors are activated and a normally itch-inhibiting subset of TRPV1$^+$/TRPA1$^+$ nociceptors is silenced (Supplementary Fig. 7). In the absence of such silencing, the combination of activation of nociceptive pathways and inhibition of itch by TRPV1 or TRPA1 expressing nociceptors will lead to pain dominating as a sensation.

Our findings suggest that primary afferent itch-generating neurons encode functionally distinct histamine and chloroquine itch pathways (Supplementary Fig. 7). In addition to revealing modality specificity and functional specialization of somatosensory afferents, our findings could also help direct development of new treatments for itch. Administration of QX-314 may be an effective treatment for pruritus caused by either histamine or non-histamine pruritogens if they are associated with sufficient activation of TRPV1 or TRPA1. Alternatively, because large-pore channels are present in both histamine- and chloroquine-sensitive pruriceptors, targeting QX-314 broadly into these fibers via co-activation of both TRPV1 and TRPA1 channels, for example by using a non-pungent TRPV1/TRPA1 co-activator[23,24,43], may have therapeutic promise for preventing or blocking both histamine-evoked itch and histamine-independent itch, although at the expense of also producing analgesia.

METHODS

Combined Calcium Imaging and Voltage Clamp recordings

Cell Culture—Trigeminal neuron cultures were prepared from adult (2 to 3-month-old) CD1 mice[44]. In short, trigeminal neurons were removed and placed into HBSS and 1% penicillin–streptomycin (Sigma), then digested in 5mg/ml collagenase, 1mg/ml Dispase II (Roche, Indianapolis, IN). Cells were triturated in the presence of DNAse I inhibitor (50U) and centrifuged through 10% BSA (Sigma). The cell pellet was resuspended in 1ml Neurobasal (Sigma) containing B27 supplement (Invitrogen), penicillin and streptomycin (Sigma), 10μM AraC. Cells were plated onto poly-d-lysine (500ug/ml) and laminin (5 mg/ml) coated 35 mm tissue culture dishes (Becton Dickinson) at 8000–9000 per dish, at 37°C, 5% carbon dioxide.

Ratiometric calcium imaging—Cultured adult trigeminal neurons were loaded for 45–60 minutes with 1 μM fura-2 AM (stock in DMSO) in a bath solution composed of (in mM): 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 Glucose, 10 HEPES and then rinsed for 45–60 minutes for de-esterification of intracellular AM esters. Neurons were perfused continuously at 2 ml/min and examined with an inverted microscope (Eclipse Ti) equipped with Epi-Fl attachment; perfect focus system (Nikon, Japan) and Exi Aqua monochromator (QImaging). Intracellular $[Ca^{2+}]_i$ was measured fluorometrically as an absorbance ratio at 340 nm and

380 nm (3F340/380) (510 nm for emission) (Lambda DG4, Sutter Instruments). Images were taken every 1 second, were monitored online and analyzed offline using Nikon Elements AR Software (Nikon). Histamine (100μM) and chloroquine (100μM) were briefly bath applied (60 s) using a fast-step valve control perfusion system (Harvard Apparatus). In all responsive neurons, the changes in ratio (ΔF) following application of histamine and chloroquine were larger than 0.1ΔF and were easily distinguishable from optic noise, which was less than 0.025ΔF. Whole cell voltage clamp recordings were then performed from the responsive cells (histamine-positive or chloroquine-positive) and non-responsive cells (histamine-negative or chloroquine-negative).

Electrophysiology—For electrophysiological recordings of transmembrane sodium currents, in order to decrease driving force for sodium the solutions were replaced after completing calcium imaging measurements to thereafter contain (in mM): 60 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 0.1 $CdCl_2$, 10 HEPES, 10 Glucose, 5 4-AP,60 Choline chloride, 15 TEA-Cl, (pH, 7.4). Pipette solution contained (in mM): 110 CsCl, 25 CsOH, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES, (pH = 7.4 with CsOH). Recordings were performed using a Multiclamp 700 B amplifier (Molecular Devices) at room temperature (22 ± 2°C). Data were low pass filtered at 1 kHz (−3 dB, 4 pole Bessel filter) and sampled at 10 kHz. Patch pipettes were pulled from thick-walled borosilicate glass capillaries (1.5 mm outer diameter, Sutter Instrument) on a Sutter Instrument P-1000 puller (Novato, CA) and had a resistance of 2–5 MΩ. Pipette potential was zeroed before seal formation and membrane potential not corrected for the small liquid junction potential (−2.2 mV). Care was taken to maintain membrane access resistance as low as possible (usually 3–7 MΩ and always less than 10 MΩ). Capacity currents were cancelled and series resistance 80% compensated. Linear leakage currents were digitally subtracted on-line using a P/4 procedure. Command voltage protocols were generated on-line with a Digidata 1200 A/D interface (Molecular Devices). Data were digitized on-line using pCLAMP 10.2 (Molecular Devices). Data averaging and peak detection were made using pCLAMP 10.2 software (Molecular Devices). Data were fitted using QPlot.

For these experiments we have used data only from neurons in which input resistance and leak current did not significantly change during 10 minutes of drug application (20 min of recording). Due to the relatively low number of responsive cells and long and complex protocol of subsequent electrophysiological recording we have used data from 3 cells per group (7 groups).

Calcium Imaging (alone) Experiments

All dissections were performed on adult (2 to 4-month-old) male CD-1 mice. The calcium-indicator dye Fura-2 AM was introduced at 2 μg/ml for 30 minutes at room temperature, washed, and analyzed on a Nikon Eclipse Ti inverted microscope with exi-aqua CCD camera with NIS-elements AR 3.10 software. Each cell was given three minutes to recover from each pruritogen exposure, and 8 minutes to recover between exposure to AITC and capsaicin. Each exposure was for sixty seconds, except for capsaicin, which was exposed only for 10 seconds. Pruritogens were locally applied, with the perfusion opening placed approximately 150 microns from the field of view, and preliminary exposure to standard extracellular solution was employed prior to exposure to reagents made in SES. Pruritogen order was assigned at random (coin toss) for each plate, and no order effects were observed between differently ordered groups.

Behavioral Studies

All animal procedures were approved by the Boston Children’s Hospital Animal Care and Use Committee. Naïve adult (2 to 4-month-old) male CD-1 mice (Charles River

Laboratories) housed in groups of 5 using a normal 12-hour light/dark cycle were used. Animals were fully habituated to handling prior to all experimental procedures and were randomly assigned to experimental groups. The day before beginning itch experiments, mice were briefly anesthetized by inhalation of 1–2% isofluorane and a ~1 cm^2 area of hair was shaved on the right cheek of each mouse. Capsaicin (Sigma-Aldrich, St. Louis MO) was freshly prepared by dilution in vehicle (20% ethanol, 5% Tween 20 in saline; 10 ml). All other drug solutions were prepared freshly in normal saline (0.9% NaCl).

Itch Assay—Mice received intradermal (id) microinjection of pruritogen, algogen, QX-314, vehicle or a combination of pruritogen or algogen together with QX-314 intradermally in the cheek. To ensure proper intradermal injection, needle puncture with a 28g needle was initiated bevel-up at 5° to the plane of taut skin until initial penetration, then inserted horizontally until the needle tip was 0.5 cm beyond the point of insertion before intradermal evacuation of syringe contents. Correct injection was confirmed by presence of a slightly domed bulla immediately following removal of needle (Supplementary Fig. 2c). For sequential injection experiments, the intradermal bullae of the conditioning (first) injection were outlined with fine-tip permanent marker to denote the extent of intradermal drug distribution, thus providing visible drug distribution boundaries for subsequent injections (Supplementary Fig. 2). Animals not receiving proper drug injections were noted and excluded from the study prior to observation. Immediately after conditioning injection, mice were placed in in a custom-built itch observation apparatus and video recorded during the mouse dark cycle as previously described[26]. Scratches and/or wipes subsequently quantified by blinded observers.

Von Frey assay—Mice were assessed manually using the up-down method to determine the median 50% (5/10) withdrawal threshold[45].

Radiant heat (Hargreaves) assay—Radiant heat withdrawal threshold was determined using the Plantar Analgesia Meter (IITC Life Science, Inc.) according to methods previously described[46].

Focal cold plantar (Brenner) assay—Latency to remove hindpaw from focally applied cold stimulus was performed according to methods previously described[27] only using 3/8″ thick (4.78 mm, as measured by electronic caliper) Neoceram™ N-0 thermal-shock-resistant glass (Nippon Electric Glass Co., Ltd.).

Adhesive dot plantar tactile (“sticky tape”) assay—Latency to bite, lick, or attempt removal of a 9 mm diameter circular adhesive Microtube Tough-Spots® label (Diversified Biotech) was performed according to methods previously described[28], only using hindpaw instead of forepaw for application of stimulus.

Statistical Analysis

Sample sizes for all experiments were chosen according to standard practice in the field. For electrophysiological experiments the significance of the effect was calculated using two-way ANOVA non-parametric test followed by Bonferroni post-test. Comparison of group means for behavior studies was performed using Student’s t-test. All bar graphs are plotted as mean ± standard error of the mean (SEM). For behavioral studies, “*n*” represents the total number of mice used in each group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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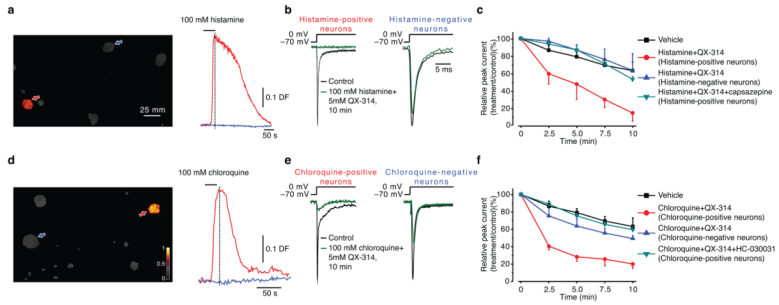

Figure 1. Application of pruritogens leads to a QX-314-mediated blockade of sodium currents selectively in pruritogen-sensitive trigeminal ganglion (TG) neurons

(a) Photomicrography (left) and representative traces (right) of changes in intracellular calcium concentration recorded from cultured trigeminal ganglia neurons at left. Red arrow indicates small trigeminal neuron that responded (red trace) to histamine (60 seconds, 100 μM). Blue arrow indicates cell that did not respond (blue trace) to application of histamine. Dotted line indicates the time point of the photomicrography frame. **(b)** Representative, normalized traces of sodium currents recorded from histamine-positive (left) and histamine-negative (right) neurons before (black) and 10 minutes after (green) application of 100 μM histamine together with 5 mM QX-314. **(c)** Time course of the changes in amplitude of peak sodium current. The specific TRPV1 antagonist capsazepine (20 μM) abolished the histamine/QX-314-mediated decrease in sodium current. Results are mean ± SEM of peak sodium current relative to control (n = 3 for each group. For statistical analysis see Supplementary Table 2). **(d)** Photomicrography (left) and representative traces (right, dotted line indicates the time point of the photomicrography frame) of changes in intracellular calcium concentration following application of chloroquine (60 seconds, 100 μM). **(e)** Representative, normalized traces of sodium current recorded from chloroquine-positive (left) and chloroquine-negative (right) neurons before (black) and 10 minutes after (green) application of 100 μM chloroquine together with 5 mM of QX-314. **(f)**. Time course of changes in amplitude of peak sodium current. The specific TRPA1 antagonist HC-030031 (100 μM) abolished chloroquine/QX-314-mediated decrease in sodium current. Results are mean ± SEM of peak sodium current relative to control (n = 3 for each group. For statistical analysis see Supplementary Table 4).

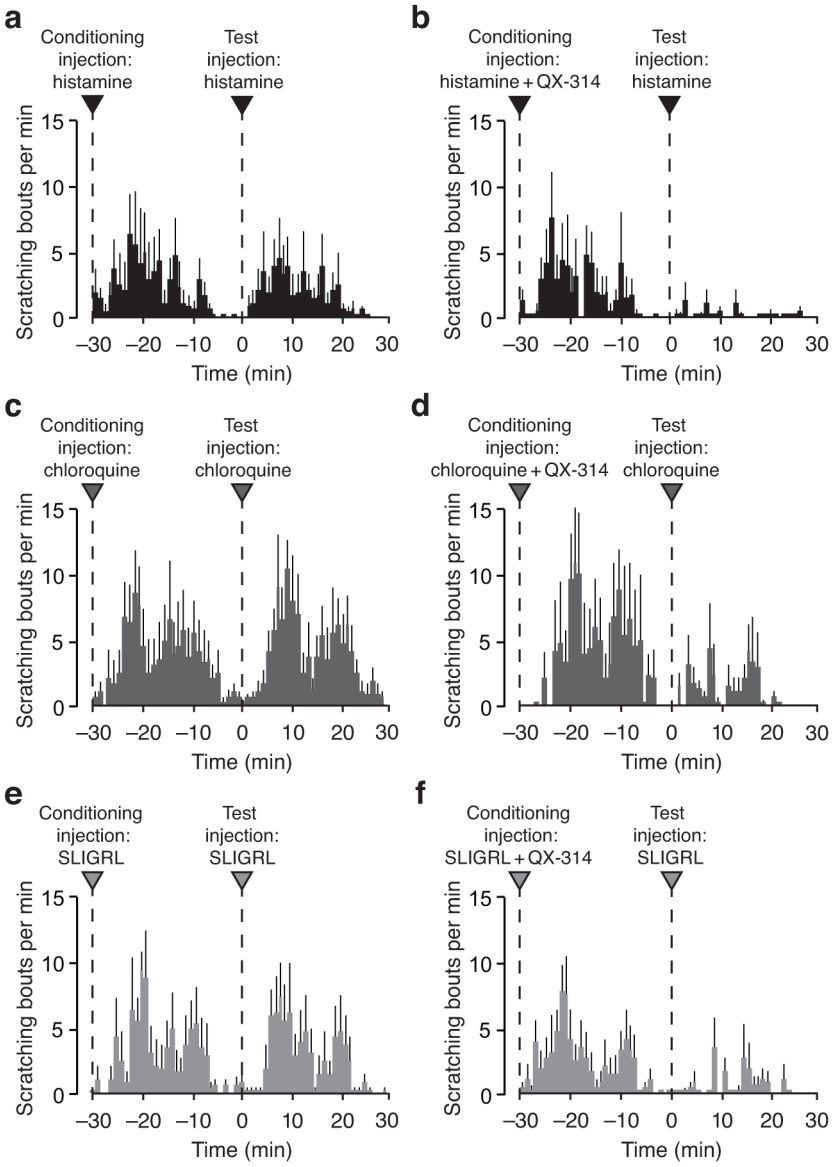

Figure 2. Co-administration of QX-314 and pruritogens inhibits subsequent pruritogen-evoked scratching

(a, c, e) Sequential pruritogen cheek injections at 30-minute inter-stimulus intervals (ISI) evoke similar levels of cheek scratching (itch) bouts. **(b, d, f)** Pruritogen-evoked scratching is inhibited 30-minutes after conditioning injection of pruritogen together with 1% QX-314. **(a)** Conditioning injection: histamine (100 μg/20 μl), total scratching bouts in 30 minutes (TSB/30 minutes) = 64.3 ± 7.5 bouts. Test injection: histamine (100 μg/10 μl), TSB/30 minutes = 49.2 ± 9.7, $P > 0.05$, n = 6, degrees of freedom (d.f.) = 10. **(b)** Conditioning injection: histamine (100 μg/20 μl) + 1% QX-314, TSB/30 minutes = 56.0 ± 8.3. Test injection: histamine (100 μg/10 μl), TSB/30 minutes = 7.5 ± 3.8, $P < 0.001$, n = 6, d.f. = 10. **(c)** Conditioning injection: chloroquine (CQ, 50 μg/20 μl), TSB/30 minutes = 103.0 ± 19.1. Test injection: chloroquine (50 μg/10 μl), TSB/30 minutes = 109.0 ± 20.8, $P > 0.05$, n = 7, d.f. = 12. **(d)** Conditioning injection: chloroquine (50 μg/20 μl) + 1% QX-314, TSB/30 minutes = 110.0 ± 13.7. Test injection: chloroquine (50 μg/10 μl), TSB/30 minutes = 31.8 ± 13.3, $P < 0.01$, n = 6, d.f. = 10. **(e)** Conditioning injection: SLIGRL (50 μg/20 μl), TSB/30

minutes = 80.8 ± 9.6. Test injection: SLIGRL (50 μg/10 μl), TSB/30 minutes = 71.4 ± 12.0, $P > 0.05$, n=5, d.f. = 8. **(f)** Conditioning injection: SLIGRL (50 μg/20 μl) + 1% QX-314, TSB/30 minutes = 71.2 ± 16.1. Test injection: SLIGRL (50 μg/10 μl), TSB/30 minutes = 17.7 ± 8.0, $P < 0.05$, n = 6, d.f. = 10. Figure results are mean ± SEM of total scratching bouts per minute for 30 minutes after conditioning injection and test injection. Total scratching bouts in 30 minutes (TSB/30 minutes) are mean ± SEM of total scratching bouts in 30 minutes following injection. P-values represent comparison of value of mean total scratching bouts in 30 minutes evoked by test injection to those evoked by conditioning injection.

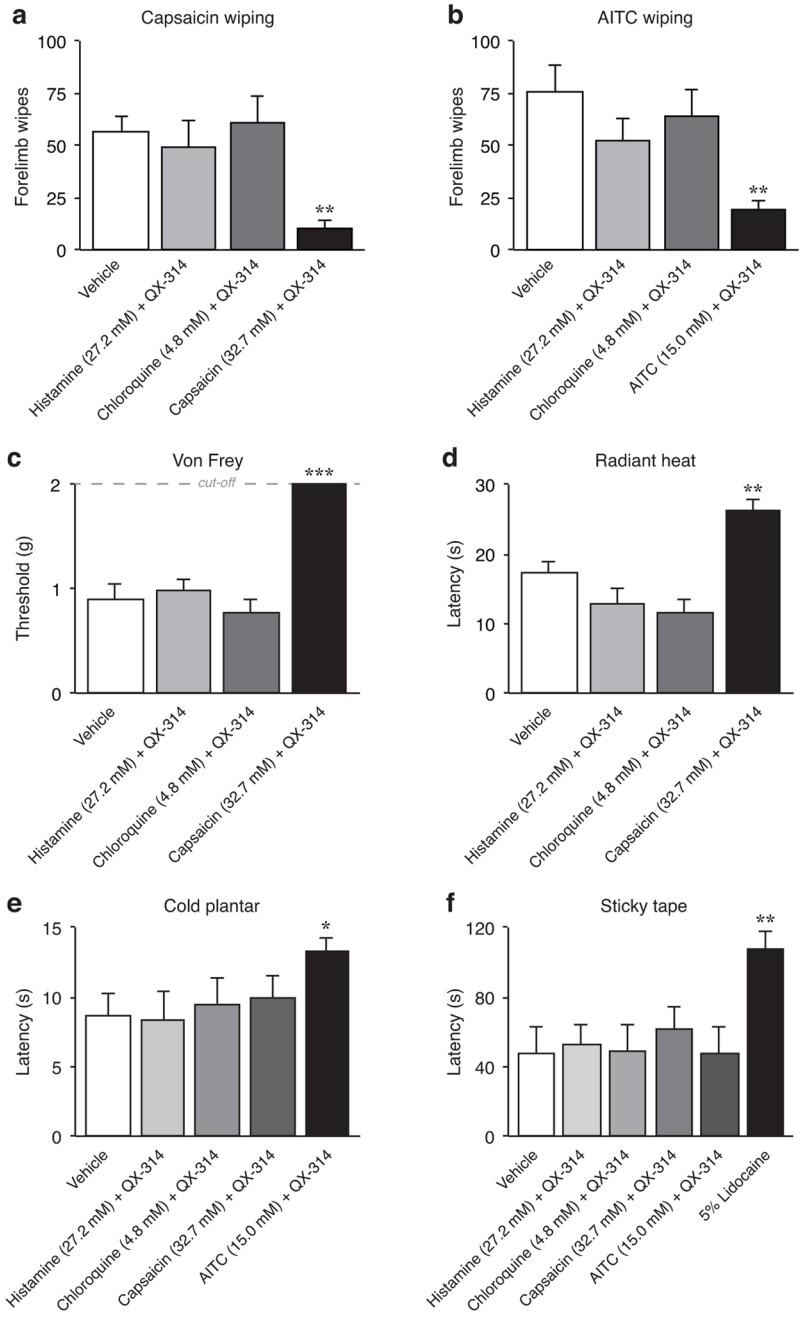

Figure 3. Silencing pruritogen-responsive neurons does not alter behavioral responses to non-itch stimuli

Forelimb wipes (pain-related behavior) evoked by **(a)** capsaicin (0.1%, 10 μl) and **(b)** AITC (0.15%, 10 μl) 30 minutes after cheek conditioning injections of vehicle or 1% QX-314 together with pruritogens (histamine, chloroquine (CQ)) or algogens (capsaicin, AITC). Hindpaw withdrawal to **(c)** punctate mechanical (von Frey) stimulus, **(d)** a radiant heat (52° C) stimulus, or **(e)** focally applied cold stimulus (see online methods) 30 minutes after intraplantar conditioning injections of vehicle or 1% QX-314 together with pruritogens or algogens. **(f)** Latency to bite, lick or attempt removal of an adhesive dot on the plantar surface of the hindpaw 30 minutes after intraplantar conditioning injections of vehicle, 1%

QX-314 together with pruritogens or algogens was compared with a group receiving saline or intraplantar injection of 5% lidocaine (after 5 minutes). P-values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$). Error bars, SEM. $n = 5–7$ for all groups.

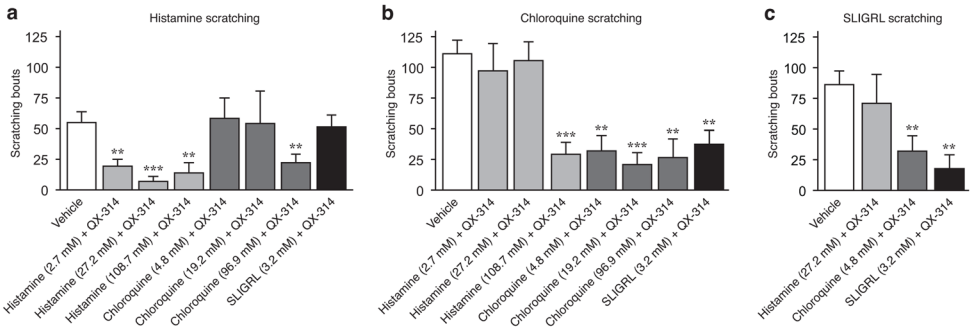

Figure 4. Distinct primary afferents mediate histaminergic itch and non-histaminergic itch

Pruritogen-evoked cheek scratching (itch) bouts 30 minutes after different conditioning injections indicated at the x-axes. **(a)** Intradermal test injection of histamine (100 μg/10 μl) alone, **(b)** chloroquine (CQ, 50 μg/10 μl) alone or **(c)** SLIGRL (50 μg/10 μl) alone 30 minutes after conditioning injection of vehicle or pruritogens together with 1% QX-314 P-values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *, $P < 0.05$; ** $P < 0.01$). Error bars, SEM. $n = 5–7$ for all groups.

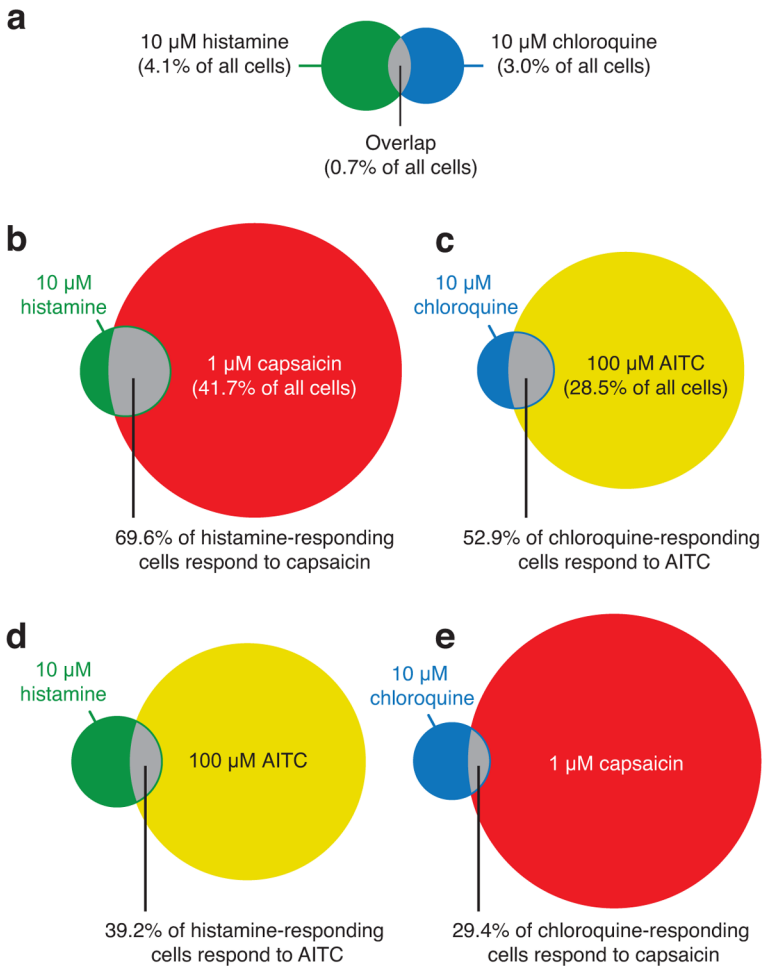

Figure 5. Proportional representation of coincident trigeminal cell responses to low dose chloroquine and histamine, and their overlapping responsiveness with capsaicin and AITC

Venn diagram of calcium responses of 564 cultured trigeminal (TG) neurons to 10 μM histamine, 10 μM chloroquine (CQ), 1 μM capsaicin, and 100 μM AITC. **(a)** Histamine (10 μM) activated 23 of 564 TG neurons, while 17 responded to 10 μM chloroquine. Among histamine-activated cells 19 of 23 responded to histamine but not chloroquine, and 4 of 23 responded to both histamine and chloroquine. For TG cells responding to chloroquine 13 of 17 responded to chloroquine but not histamine. **(b)** Capsaicin activated 235 of 564 cells. Among histamine responsive cells, 16 of 23 also responded to capsaicin. **(c)** AITC activated 161 of 564 TG neurons, and more than half of chloroquine-responding cells (9 of 17) responded to AITC. **(d)** AITC activated 9 of 23 of histamine-responding cells. **(e)** Capsaicin activated 5 of 17 of chloroquine-responsive TG neurons.

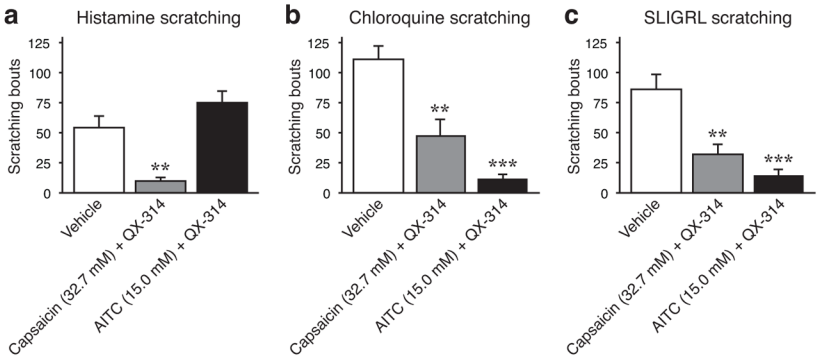

Figure 6. Selective silencing of nociceptor populations differentially inhibits histamine itch and non-histamine Itch

Cheek scratching (itch) following intradermal test injection of (**a)** histamine (100 μg/10 μl) alone ($n = 6$), **(b)** chloroquine (CQ, 50 μg/10 μl) alone ($n = 6–7$), and **(c)** SLIGRL (50 μg/10 μl) ($n = 5–6$) 30 minutes after a conditioning injection of vehicle (0.9% NaCl, 20 μl) or 1% QX-314 together with capsaicin or AITC. P-values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$). Error bars, SEM.

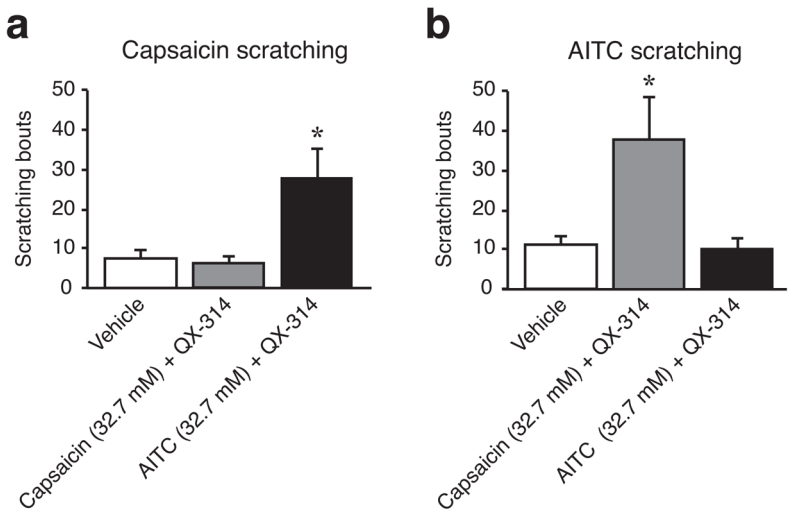

Figure 7. TRPV1$^+$/TRPA1$^+$ fibers suppress itch

(a) Hindlimb scratching (itch) evoked by capsaicin (0.1%, 10 μl) thirty-minutes after intradermal injection of vehicle (0.9% NaCl, 20 μl), capsaicin (0.1%) together with 1% QX-314 (20 μl) or AITC (0.15%) together with 1% QX-314 (20 μl). Note the significant increase in capsaicin-mediated scratching following application of AITC with QX-314. **(b)** Thirty-minutes after injection of vehicle (0.9% NaCl, 20 μl) intradermal cheek injection of AITC (0.15%, 10 μl) generated little hindlimb scratching over the next 30 minutes and this was not changed following a conditioning injection of AITC together with 1% QX-314. However, the AITC evoked scratching increased following intradermal cheek injection of capsaicin (0.1%) together with 1% QX-314 (20 μl). P-values represent comparison to vehicle value (not significant, $P > 0.05$; *, $P < 0.05$). Error bars, SEM. $n = 6$ for all groups.